DIFFERENTIAL RESPONSE OF ATP AND ORTHOPHOSPHATE IN CYTOSOL AND MITOCHONDRIA OF RAT HEPATOCYTES TO TREATMENT WITH P_i AND D-GALACTOSAMINE

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1. Introduction

In isolated rat hepatocytes a close correlation was observed between the intracellular level of orthophosphate and the rate of glycogenolysis and glucose output, respectively [1]. The intracellular P_i level could be increased by addition of orthophosphate to the incubation medium and decreased by D-galactosamine. The inverse effects of D-galactosamine and P_i on glycogenolysis indicated a phosphate-limited activity of phosphorylase *a* (EC 2.4.1.1) in the intact hepatocyte. The enzyme system involved in glycogen degradation and glucose release is thought to be localized in the extramitochondrial space of the cells. Using the digitonin method [2], it was attempted to study separately the response of the cytosolic and the mitochondrial P_i and ATP contents of isolated hepatocytes treated with orthophosphate and D-galactosamine, respectively. It will be shown that these compounds react differently in the two compartments and that the changes elicited in the cytosol are correlated to the observed rates of glycogenolysis.

Abbreviations: GalN, D-galactosamine; P_i, orthophosphate

Preliminary accounts of this work were presented at the Spring Meeting of the Gesellschaft für Biologische Chemie in Frankfurt, March 1978, and the 12th FEBS Meeting in Dresden, July 1978

2. Materials and methods

Isolated rat hepatocytes were prepared and incubated as in [1]. Cytosolic and mitochondrial fractions of hepatocytes were separated by the digitonin method in [2] with slight modifications (F. Hofmann, personal communication): After incubation at 37°C, the cells were centrifuged at 4°C for 2 min at 3000 × *g*. The pellet was rapidly mixed on a Whirli mixer with 1 ml ice cold 0.3 M triethanolamine·HCl buffer (pH 7.0), containing 0.25 M sucrose, 3 mM EDTA and 0.2% digitonin (w/v). After 40 s, the suspension was centrifuged for 10 s (Eppendorf centrifuge 3200) at 8000 × *g* and 4°C. For measuring the enzyme activities, the mitochondrial pellet was quickly frozen in liquid nitrogen. After thawing the pellet was suspended and homogenized in 1 ml triethanolamine–sucrose–EDTA buffer. The supernatant was used without further treatment for activity determinations.

Metabolites were assayed both in the pellet and in the supernatant fraction after deproteinization with $HClO_4$. To the cytosolic fraction (1 ml) 100 µl 2.9 N $HClO_4$ were added at once. The particulate fraction was quickly frozen in liquid nitrogen. After addition of 600 µl 0.6 N $HClO_4$, the frozen pellets were thawed with shaking and homogenized in the presence of 2% (w/v) Triton X-100. After removal of protein by centrifugation, the extracts were neutralized with potassium hydroxide and recentrifuged.

Glucose [3], ATP [4] and orthophosphate [5] were determined enzymatically. Lactate dehydrogenase

(EC 1.1.1.27) was assayed by the method in [6] and glutamate dehydrogenase (EC 1.4.1.2) as in [7].

3. Results and discussion

The separation of the cytosolic and particulate fraction was checked by measuring the activities of lactate dehydrogenase and glutamate dehydrogenase; > 95% of lactate dehydrogenase and < 5% of glutamate dehydrogenase activity were found in the cytosolic fraction.

The distribution of ATP and P_i between cytosol and mitochondrial pellet is shown in table 1. The sum of mitochondrial and cytosolic ATP corresponds well with the ATP content of hepatocytes as determined in whole cells, thus excluding loss of conversion of metabolites during the separation procedure. In particular, the P_i contents of either cell compartment do not seem to be distorted by hydrolysis of nucleoside triphosphates during the separation.

A correlation between the P_i content and the rate of glycogenolysis in isolated hepatocytes should be seen most clearly in the cytosolic fraction. The determination of P_i levels in the separated compartments after various treatments of the hepatocytes supported this conclusion. Addition of P_i during the incubation of the cells drastically increased the cytosolic P_i content without appreciably changing the mitochondrial level (fig.1). On the other hand, pretreatment of the cells with GalN lowered within 10 min the P_i content in both compartments.

Table 1
Distribution of ATP and P_i in isolated rat hepatocytes

Fraction	ATP (mmol . (kg wet wt)$^{-1}$)	P_i
Intracellular (total)	2.29 ± 0.20	3.94 ± 0.49
Cytosolic	1.72 ± 0.15	1.52 ± 0.35
Mitochondrial	0.53 ± 0.14	2.10 ± 0.25
Cytosolic + mitochondrial	2.25 ± 0.18	3.62 ± 0.52
% Total	98	92

Cells, 50 mg, were incubated in Umbreit/Ringer/carbonate buffer (pH 7.4) containing 0.4 mM P_i. After 5 min, the cells were fractionated as in section 2. The total intracellular contents were obtained by immediate acid treatment of the harvested cells [1]. Data are given as means ± SD of 7 independent observations

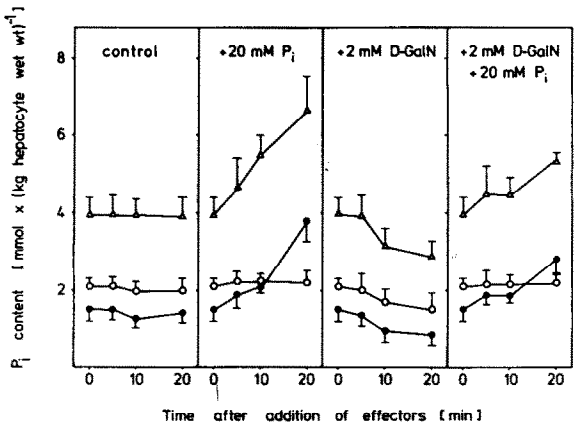

Fig.1. Response of cytosolic and mitochondrial P_i levels of isolated hepatocytes to addition of P_i and D-galactosamine, respectively. The separation of the subcellular fractions and the assays are in section 2. Each point is the mean ± SD of 7 independent experiments. (△) Whole cells; (●) mitochondrial pellet; (○) cytosolic fraction.

The results support the proposition [1] that the actual activity of phosphorylase *a* in isolated rat hepatocytes is limited by the cytosolic P_i concentra-

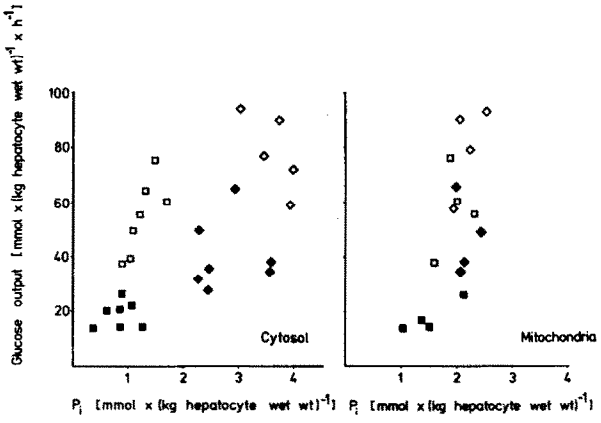

Fig.2. Correlation between rate of glucose formation and P_i level of the cytosolic and mitochondrial compartment, respectively. (□) Untreated cells; (◇) with 20 mM P_i; (■) with 2 mM D-galactosamine; (◆) with 20 mM P_i and 2 mM D-galactosamine in the medium. Glucose output was calculated between 10–20 min after addition of the effectors, the P_i values are those after 20 min.

tion; assuming an aqueous cytosolic space of 0.5 ml/g liver wet wt [8], P_i would be ~3 mM which is below the saturation level of the enzyme [1,9]. The P_i-stimulated and GalN-inhibited rate of glucose output is correlated to the cytosolic rather than to the mitochondrial P_i content (fig.2).

Addition of P_i to isolated hepatocytes was shown [1] to increase also the intracellular level of ATP. This rise is practically confined to the cytosolic space (fig.3). GalN (2 mM) added to the incubation medium lowered the level of ATP both in the cytosol and in the mitochondria by 40–50%. During these experiments, the sum of acid-soluble adenine nucleotides was not altered (data not shown) excluding a stimulated degradation or synthesis as possible mechanism for the changing ATP concentration. The simultaneous presence of 20 mM P_i in the incubation medium prevented the GalN-mediated decrease of the intracellular ATP content (fig.3).

The data indicate that during the first 20 min, the mitochondrial P_i and ATP contents are barely affected by increasing levels in the cytosol but that they immediately follow a decrease in the extramitochondrial compartment. This differential behaviour is not only of importance for the cellular response to phosphate-trapping agents [1,10,11], but may also be of regulatory significance under conditions of enhanced or inhibited P_i transport.

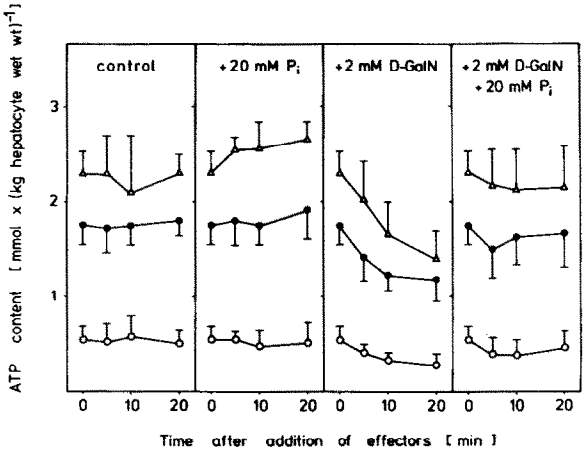

Fig.3. Response of the cytosolic and mitochondrial ATP levels of isolated hepatocytes to addition of P_i and D-galactosamine, respectively. The separation of the subcellular fractions and the assay procedures are in section 2. Each point represents the mean ± SD of 7 independent determinations. (△) Whole cells; (●) mitochondrial pellet; (○) cytosolic fraction.

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